Exhibit A

Response of Bladder Carcinoma Cells to TRAIL and Antisense Oligonucleotide, Bcl-2 or Clusterin Treatments

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Purpose: Bladder transitional cell carcinoma is the second most common urological malignancy, of which 80% are superficial disease limited to the bladder. Superficial bladder transitional cell carcinoma has a high propensity for recurrence and progression after initial resection, necessitating adjuvant intravesical therapy. TRAIL (tumor necrosis factor-related apoptosis inducing ligand) can selectively induce apoptosis in most tumor cells while sparing normal cells. TRAIL drives not only the death receptor pathway, but also the mitochondrial pathway through Bid. Due to the anti-apoptotic functions of Bcl-2 and clusterin on the mitochondrial apoptotic pathway the effects of down-regulating these proteins were examined in partially TRAIL resistant bladder transitional cell carcinoma cell lines.

Materials and Methods: Antisense oligonucleotides targeting Bcl-2 and clusterin were used alone or combined with TRAIL and cytotoxicity was examined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide) proliferation assay. Apoptotic pathway signals were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis/Western blotting after the various combination treatments. All statistical tests were 2 sided.

Results: Although no direct correlation between TRAIL sensitivity and the relative expression levels of Bcl-2 and clusterin was found in the bladder transitional cell carcinoma cell lines examined, antisense oligonucleotide mediated the down-regulation of Bcl-2 and clusterin, increasing the sensitivity of the partially resistant cells to TRAIL. This was mediated through increased apoptotic signaling of the mitochondrial pathway, as evident by the increased activation of caspase-9 and 3, and cleaved DFF45. There was no benefit of combined antisense oligonucleotide therapy.

Conclusions: This study provides proof of principle that TRAIL combined with antisense oligonucleotide-Bcl-2 may have potential as a novel future treatment strategy for bladder transitional cell carcinoma.

Key Words: urinary bladder; carcinoma, transitional cell; apoptosis; tumor necrosis factors; genes, bcl-2

Abbreviations and Acronyms

ASO = antisense oligonucleotide
BCG = bacillus Calmette-Guerin
CD = cell death
Clus = clusterin
DFF = DNA fragmentation factor
DR = death receptor
MM = mismatch
MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide
TCCB = transitional cell carcinoma of the bladder
TNF = tumor necrosis factor
TRAIL = TNF-related apoptosis inducing ligand

Submitted for publication July 18, 2008.

Supported by the Canadian Cancer Society through National Cancer Institute of Canada Grant 15105.

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† Financial interest and/or other relationship with Quest Pharmatech and Nat Immune.

SUPERFICIAL TCCB has a high propensity to recur in the bladder. Intravesical BCG immunotherapy after initial tumor resection has the best response rate (70%) and it is considered the treatment of choice.[1] However, the nonspecific immune response appears to only delay the inevitable relapse with significant risks and side effects. Recent investigations have implicated the TNF family member TRAIL in BCG anti-

0022-5347/09/1813-1361/0
THE JOURNAL OF UROLOGY®

Vol. 181, 1361-1371, March 2009
Printed in U.S.A.
DOI:10.1016/j.juro.2008.10.148

tumor effects.[2] Because TRAIL activity is highly selective for tumor cells while it spares normal cells, it is clinically attractive for direct intravesical and possibly systemic application. However, despite its high treatment potential monotherapeutic approaches with TRAIL often do not effectively activate apoptosis due to the adaptive resistance of many cancer cells. Consequently investigators continue efforts to develop combination strategies that will augment TRAIL effects in these resistant cells.

The etiology of cancer cell resistance to TRAIL apoptosis is complex and attributable to a number of factors, including high expression of anti-apoptotic signaling molecules. These factors are potential candidates for therapeutic intervention. Bcl-2 and Clus are 2 anti-apoptotic factors that are implicated in cancer cell TRAIL resistance.[3,4] Bcl-2 over expression blocks TRAIL apoptosis in cancer cells[5] and its expression in TCCB is prognostic.[6] Clus, which is unrelated to Bcl-2, can exist in secreted and intracellular forms, and it is a key mediator of TRAIL resistance in prostate tumor cells.[4] Also, Clus protects various cell types from TNF-α and doxorubicin mediated cytotoxicity extracellularly.[7] We explored combinational approaches to reactivate TRAIL apoptosis in partially resistant TCCB cells by ASO inhibition of Bcl-2 and Clus proteins. We also examined factors that mediate activation of the extrinsic and intrinsic apoptotic pathways.

MATERIALS AND METHODS

Cells and Culture Conditions

The F2P6 benign fibroblast cell line and certain human TCCB cell lines were used in the study, including 5637, HT-1376 (American Type Culture Collection, Manassas, Virginia), UMUC-3, 6, 9 and 14, RT-4, 253J, T24 RT-112 and MGH-U3. Cells were grown in a humidified incubator under standard conditions of 37C and 5% CO_2 in modified Eagle's medium (with L-Glu nonessential amino acids-Earle's balanced salt solution), RPMI-1640 or Dulbecco's modified Eagle's medium, supplemented with 10% heat inactivated fetal bovine serum except where noted, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin-B. Human recombinant, nontagged soluble TRAIL (PeproTech®) was

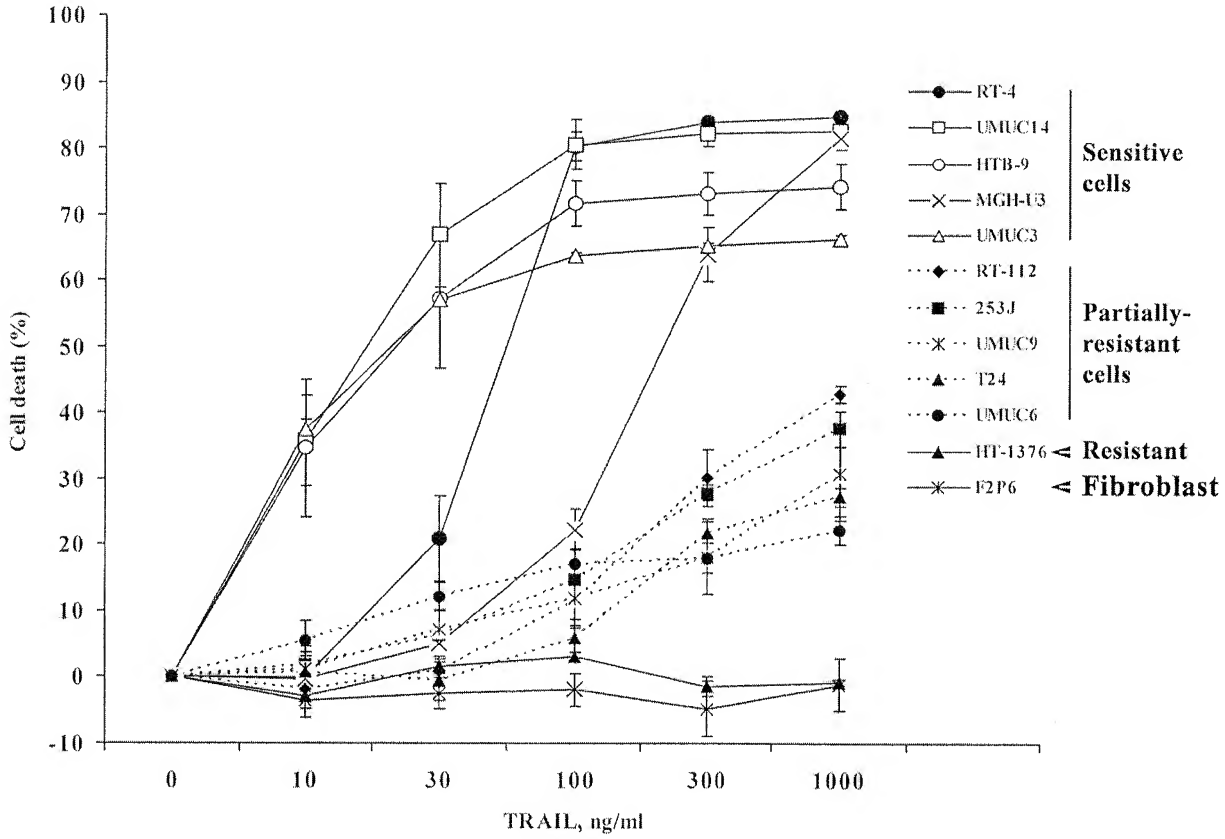

Figure 1. MTT assay shows response of human TCCB and normal fibroblast cells to culture for 24 hours in presence of 0 to 1,000 ng/ml TRAIL, as described. Each experiment had multiple replications and was repeated at least 3 times. RT-4, UMUC14, HTB-9, MGH-U3 and UMUC3 were categorized as TRAIL sensitive cell lines, RT-112, 253J, UMUC9, T24 and UMUC6 were partially resistant to TRAIL and HT-1376 was TRAIL resistant. Normal fibroblast F2P6 control cells were also refractory to TRAIL. Data are shown as proportion of nonviable cells expressed as percent of control cells.

prepared as a stock solution in sterile distilled water and stored at −20C.

Cytotoxicity Assays

Exponentially growing cells (1×10^4) in 96-well plates were incubated with control medium or medium supplemented with escalating concentrations of TRAIL for 24 hours. Cell viability was estimated with 20 μl 5 mg/ml MTT and the resultant formazan crystals were dissolved in 150 μl dimethyl sulfoxide. The absorbance of each well was measured with a microplate reader at 570 nm and percent CD was calculated as (1 − [absorbance of treated cells/absorbance of untreated cells]) × 100. Each assay was repeated at least 3 times.

ASO Transfection

TCCB cells (approximately 4.5×10^5) cultured overnight in antibiotic-free medium were transfected with phosphorothioated Clus and/or Bcl-2 ASO using Lipofectin®. Briefly, 20 μl (100 μM) ASO-Bcl-2 and/or ASO-Clus were mixed with 350 μl Opti-MEM® in an Eppendorf tube. Lipofectin reagent (7 μl) was diluted into 23 μl Opti-MEM in a second set of tubes and incubated at room temperature for 30 minutes. The ASO/Opti-MEM and the Lipofectin/Opti-MEM mixtures were combined (ASO-Lipofectin complex) and incubated for additional 20 minutes. After washing and replenishment with 1.6 ml Opti-MEM 400 μl ASO-Lipofectin complex were added to each dish (final concentration 1,000 nM) and cells were incubated for 4 hours at 37C. Transfection reagent was replaced with 3 ml antibiotic-free, fetal bovine serum supplemented medium and the cells were cultured an additional 20 hours before being treated with TRAIL (300 ng/ml) or harvested for Western blot analysis. In some experiments successive ASO transfections on days 2 and 3 were performed. In all experiments MM oligodeoxynucleotides were also transfected into cells as the transfection control.

Live/Dead Assay

Cells (3×10^4 per well) in 24-well plates were transfected with ASO-Bcl-2 and/or ASO-Clus as described and treated with TRAIL (300 ng/ml) for 4 hours. Cells were further cultured in renewed antibiotic-free medium for another 20 hours and then stained with 7.5 μM SYTO® 16 and 2.5 μM propidium iodide for 1 hour at 37C. Stained cells were examined with fluorescein isothiocyanate and Cy3 filters on an Axioplan upright digital imaging microscope (Carl Zeiss, Jena, Germany) equipped with a SenSys cooled charge coupled device camera (Photometrics, Tucson, Arizona) using an infinity corrected EC Plan Neofluar® 10× objective and MetaMorph® software.

Western Blot Analysis

Cells were washed in ice-cold phosphate buffered saline and lysed at 4C in lysis buffer. After determining the protein content of the extracts by bicinchoninic acid assay (Pierce, Rockford, Illinois) 40 μg protein were electrophoresed in 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by electroblotting onto nitrocellulose. Blots were immunostained with primary antibodies to caspase-9 and 3 (Cell Signaling Technology®), and DFF45/ICAD (Stressgen®), diluted with 5% nonfat milk to 1:1,000. Primary antibodies were detected with horseradish-peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, Pennsylvania) using echochemiluminescence detection reagents (Amersham, Little Chalfont, United Kingdom).

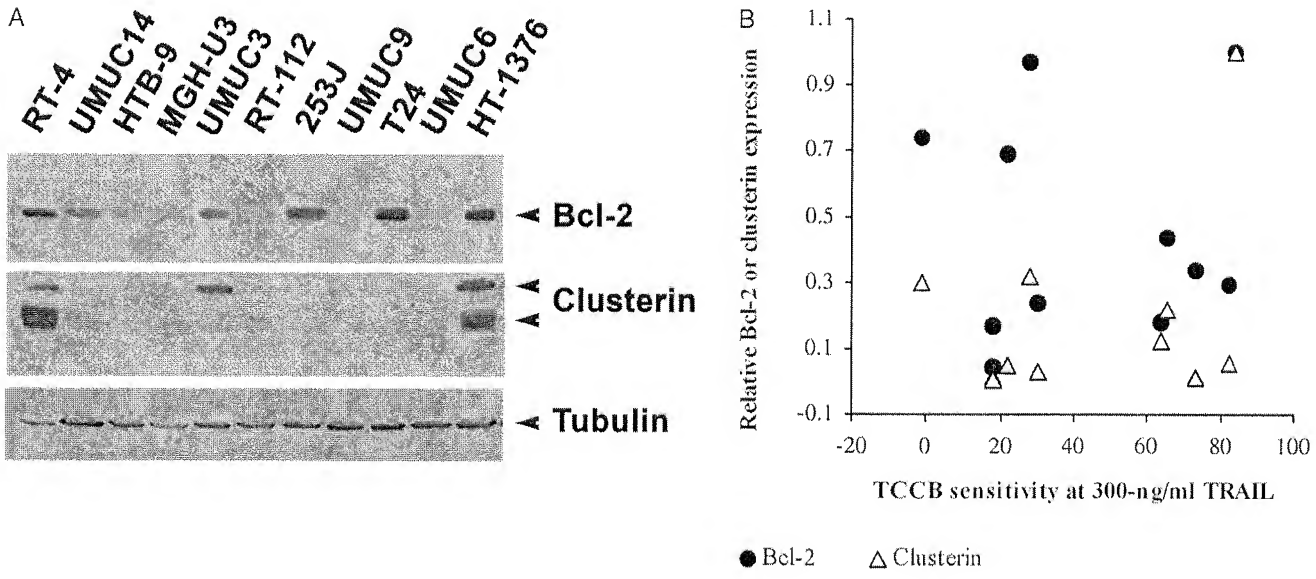

Figure 2. Relative expression of Bcl-2 and Clus proteins in human TCCB and normal fibroblast cell lines. *A*, TCCB cell lines were seeded and cultured overnight in regular growth medium. Whole cell extracts were analyzed by Western blot for Bcl-2 and Clus expression. *B*, normalized Bcl-2 and Clus level in each TCCB cell line vs corresponding sensitivity to 300 ng/ml TRAIL value in figure 1 (Bcl-2 and Clus coefficient of correlation 0.009 and 0.33, respectively).

Statistics

All data are presented as the mean ± SE of at least 3 independent experiments. Associations between the cell expression of Bcl-2 and Clus proteins, and their sensitivity to TRAIL were analyzed using the Spearman p correlation. Differences in protein expression and viability between groups were analyzed with ANOVA and the Student-Newman-Keuls multiple comparisons test using GraphPad® Prism® 3.00. All statistical tests were 2-sided with significance considered at $p \leq 0.05$.

RESULTS

TRAIL Sensitivity and Baseline Expression of Bcl-2 and Clus Proteins in TCCB Cells

The MTT assay was used to analyze the cytotoxic response in cultured TCCB and normal fibroblast (F2P6) cells after stimulation with TRAIL. TCCB cells showed differential responses, whereas F2P6 cells were completely refractory (fig. 1). The lack of a TRAIL response in F2P6 cells was consistent with findings in prior studies of the selectivity of TRAIL and its potential clinical application. Based on the cytotoxicity response we categorized the cell lines into 3 groups, including sensitive—CD greater than 65% (RT-4, UMUC-3, UMUC-14, HTB-9[5637] and MGH-U3), partially resistant—CD 10% to less than 45% (RT-112, 253J, UMUC-9, T24 and UMUC-6) and resistant or refractory—CD less than 3% (HT-1376). TRAIL induced approximately 80% CD in the sensitive group (fig. 1). In these cell lines the maximum (limiting) TRAIL response was achieved at a concentration of 100 ng/ml. This asymptotic behavior

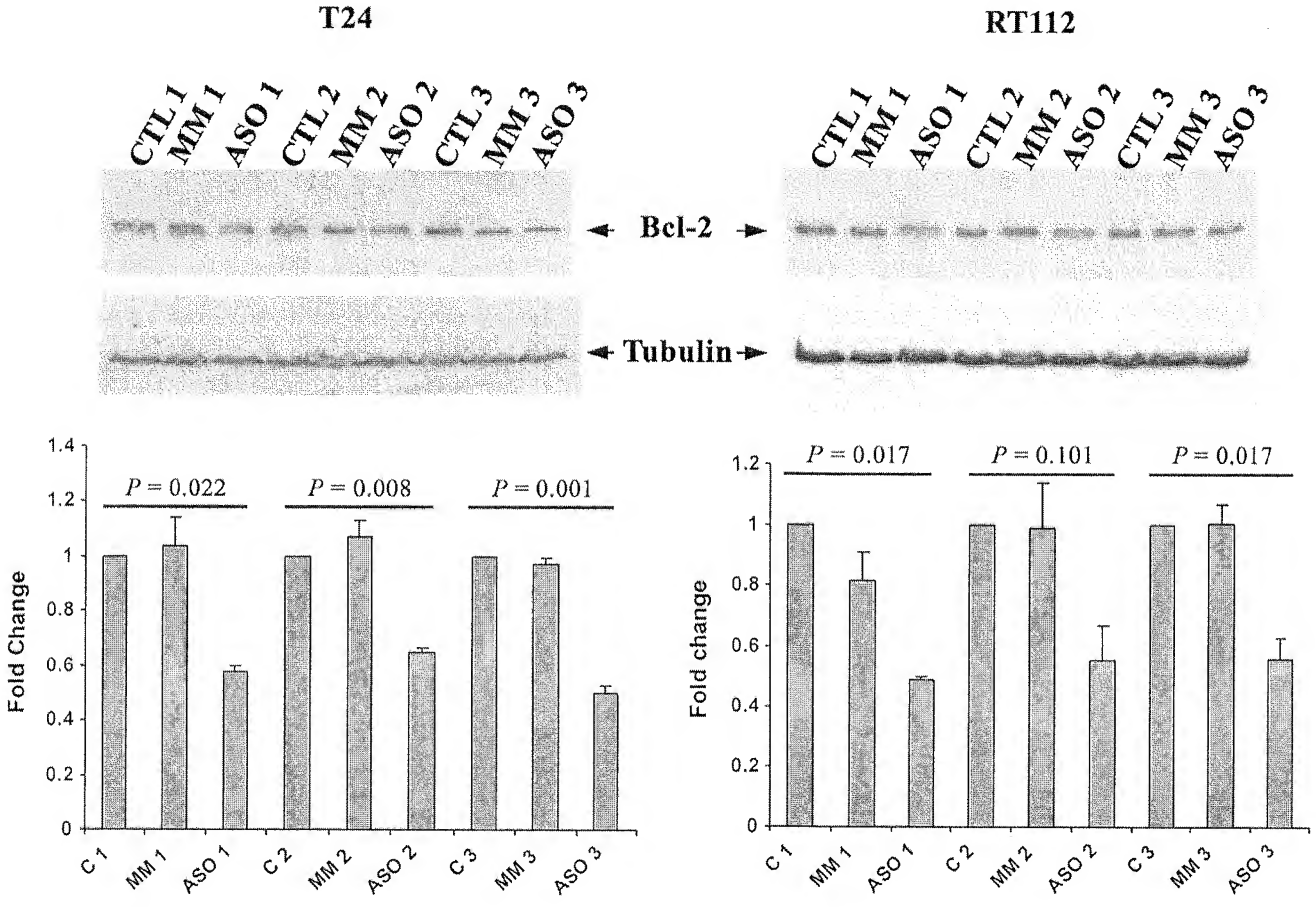

Figure 3. Effect of single vs multiple ASO-Bcl-2 transfection on Bcl-2 expression. T24 and RT112 cells were transfected with 1,000 nM phosphorothioated ASO-Bcl-2 for 4 hours per day along with MM transfection control. In single transfection experiment for 1 day in control (*CTL1*), MM (*MM1*) and ASO (*ASO1*) preparations cells were transfected with 1,000 nM phosphorothioated ASO-Bcl-2 for 4 hours, followed by culture in fresh antibiotic-free growth medium for additional 20 hours. Whole cell extracts were isolated for Western blot analysis of Bcl-2 expression. In multiple dosing experiments cells were treated for 4 hours per day for 2 consecutive days in control (*CTL2*), MM (*MM2*) and ASO (*ASO2*) preparations, and for 3 consecutive days in control (*CTL3*), MM (*MM3*) and ASO (*ASO3*) preparations using the same protocol as for single transfection before Western blot. Experiments were performed in triplicate. Bar graphs show mean ± SE quantitative analysis of Bcl-2 after normalization to tubulin.

was not observed in partially resistant cell lines, which showed only a moderate (10% to 20%) response at the 100 ng/ml mark. The refractory TCCB cell lines typified by HT-1376 showed little or no response independent of the TRAIL concentration.

To examine the relative effect of expression levels of Bcl-2 and Clus on the TRAIL response cell extracts from untreated cell lines were quantitated by Western blotting (fig. 2, *A*). Five cell lines demonstrated high Bcl-2 expression, of which 3 co-expressed high Clus levels. Figure 2, *A* shows the cell lines arranged from left to right according to the TRAIL response with RT4 the most and HT1376 the least sensitive line. These 2 cell lines coincidentally co-expressed high levels of Bcl-2 and Clus, suggesting the lack of a direct association between baseline levels of the proteins and TRAIL sensitivity (fig. 2, *B*).

Down-Regulation of Bcl-2 and Clus Expression, and Effect on TRAIL Sensitivity

It is known that Bax regulated mitochondrial apoptosis is inhibited by Bcl-2 and Clus proteins. In our study the levels of these proteins did not correlate with the TCCB TRAIL response, leading us to speculate that such an expression level possibly represents the minimum threshold for cell functioning which, if depleted, would augment the TRAIL response. To test this hypothesis we used phosphorothioated ASO to down-regulate Bcl-2 and Clus expression in an attempt to reactivate the intrinsic apoptotic pathway. We examined whether multiple ASO dosing would induce the maximal effect. Partially TRAIL resistant T24 and RT-112 cells were transfected once (4 hours per day) or on consecutive days (2 and 3). Bcl-2 expression in these cells was significantly (50%) decreased after a single ASO-Bcl-2 treatment (fig. 3).

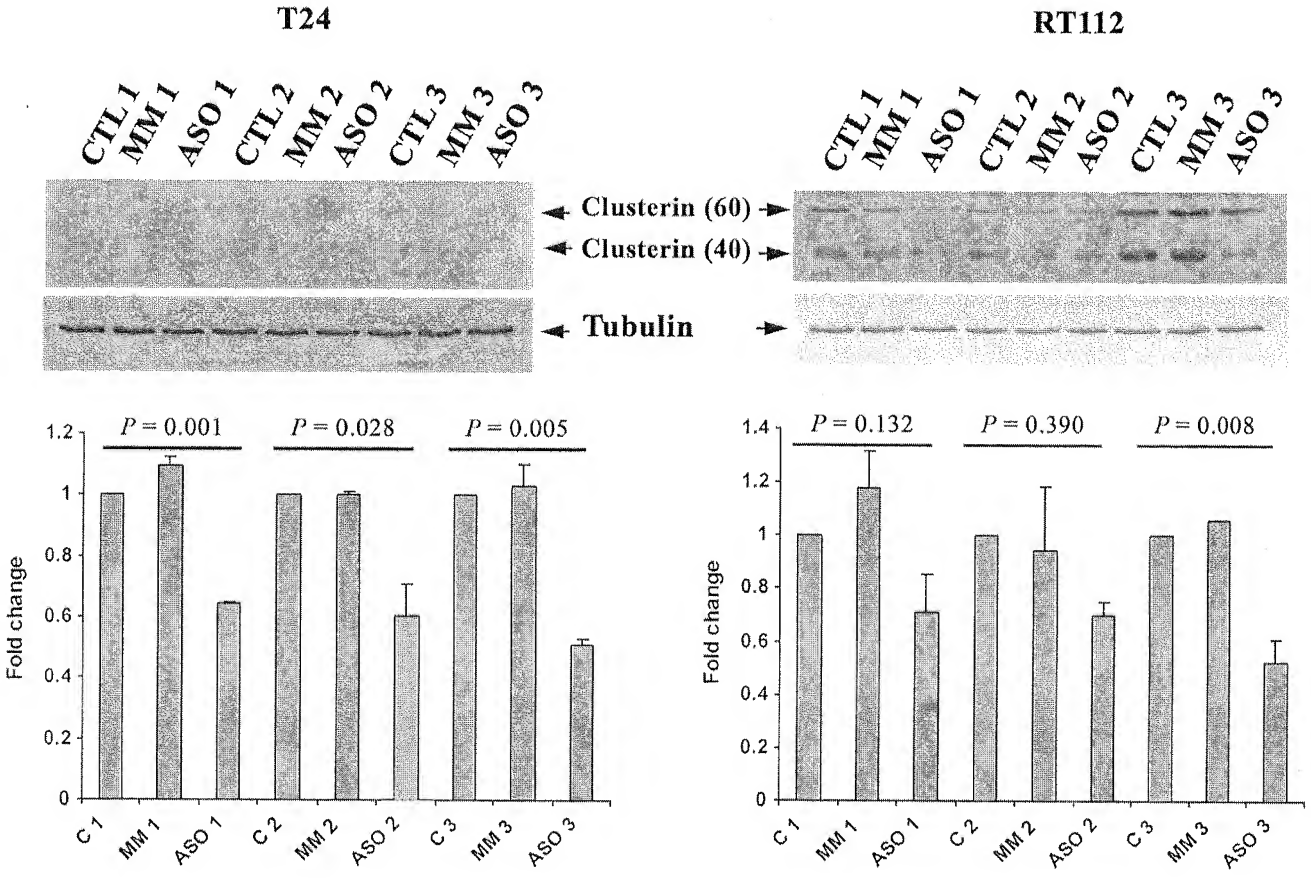

Figure 4. Effect of single vs multiple transfection of ASO-Clus on TCCB Clus expression. T24 and RT112 cells were transfected with 1,000 nM phosphorothioated ASO-Clus for 4 hours per day along with MM transfection control. In single transfection experiment for 1 day in control (*CTL1*), MM (*MM1*) and ASO (*ASO1*) preparations cells were transfected with 1,000 nM of phosphorothioated ASO-Bcl-2 for 4 hours, followed by culture in fresh antibiotic-free growth medium for additional 20 hours. Whole cell extracts were isolated for Western blot analysis of Clus expression. In multiple dosing experiments cells were treated for 4 hours per day for 2 consecutive days in control (*CTL2*), MM (*MM2*) and ASO (*ASO2*) preparations, and for 3 consecutive days in control (*CTL3*), MM (*MM3*) and ASO (*ASO3*) preparations using same protocol as for single transfection before Western blot. Experiments were performed in triplicate. Bar graphs show mean ± SE quantitative analysis of Clus expression after normalization to tubulin.

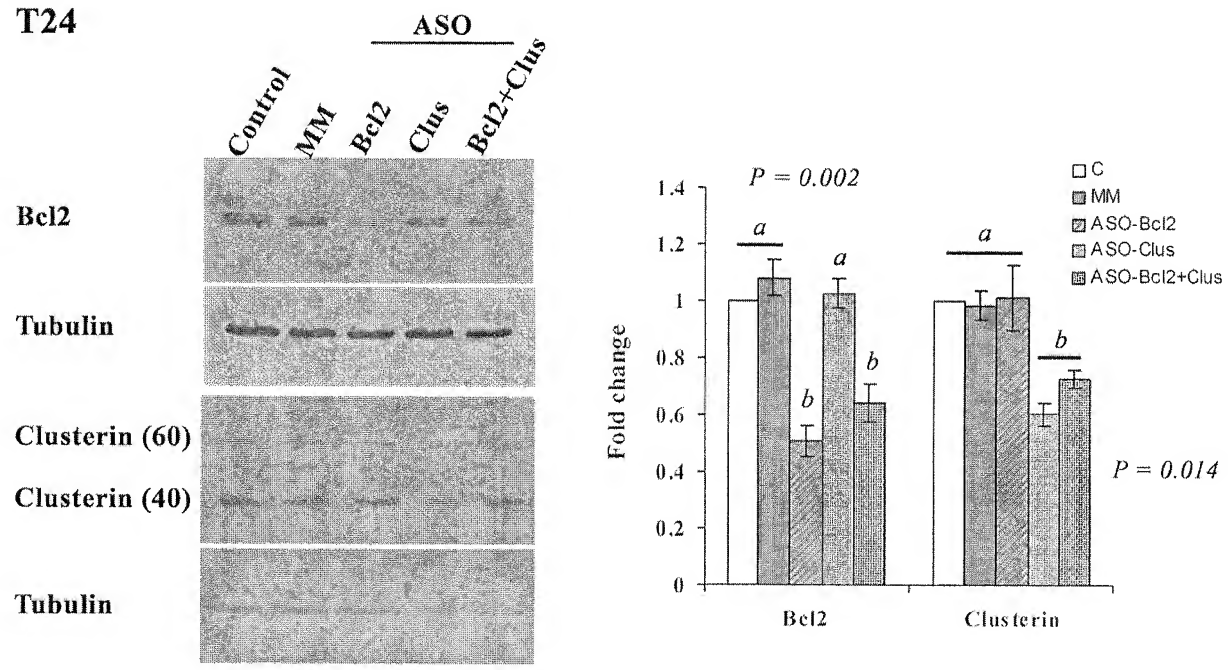

Figure 5. Protein expression of Bcl-2 and Clus after combined antisense therapy in TCCB cells. T24 cells were transfected for 4 hours with 1,000 nM ASO-Bcl2 or ASO-Clus singly or in combination as ASO-Bcl2 plus Clus, followed by additional 20-hour culture in fresh growth medium. Whole cell extracts were analyzed for Bcl-2 and Clus by Western blot. Graph shows quantitative analysis of expression of each protein after normalization to tubulin. Bars represent mean ± SE values of at least 3 separate experiments. *C*, control. *a* and *b*, significantly different.

Depletion of Bcl-2 protein after 2 or 3 consecutive transfections was proportionally similar to that of the single transfection. ASO-Clus also decreased Clus protein expression (60%) in the 2 cell lines with little or no difference between single vs multiple ASO-Clus treatments (fig. 4).

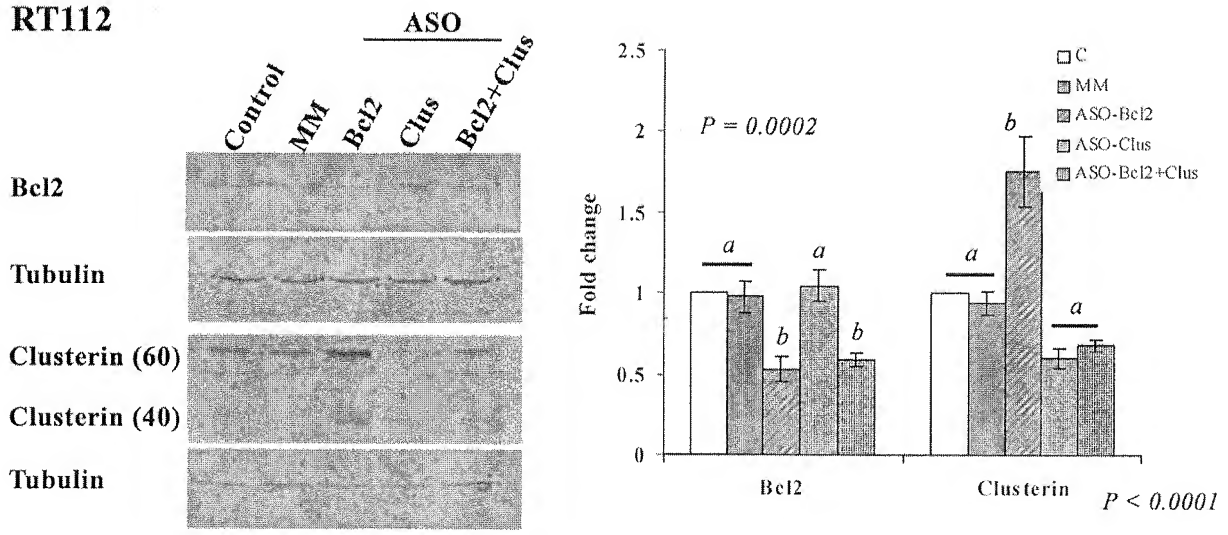

Figure 6. Protein expression of Bcl-2 and Clus after combined antisense therapy in TCCB cells. RT112 cells were transfected for 4 hours with 1,000 nM ASO-Bcl2 or ASO-Clus singly or in combination as ASO-Bcl2 plus Clus, followed by additional 20-hour culture in fresh growth medium. Whole cell extracts were analyzed for Bcl-2 and Clus by Western blot. Graph shows quantitative analysis of expression of each protein after normalization to tubulin. Bars represent mean ± SE values of at least 3 separate experiments. *C*, control. *a* and *b*, significantly different.

We then examined whether combining the 2 ASOs could lead to functional synergy in further decreasing survival protein expression. In T24, RT-112 and 253J cells ASO-Bcl-2 significantly decreased Bcl-2 expression by about 50% within 24 hours of transfection (figs. 5 to 7). This was accompanied by an increase in Clus expression except in T24. Also, ASO-Clus significantly decreased Clus expression in these cell lines (RT-112 not significant) but with no effect on Bcl-2. Aside from these observations we found no evidence of functional synergy with combined ASO therapy because the magnitude of combination treatment effects was similar to that of single therapeutic effects.

After we established the effects of the Bcl-2 and Clus reduction we examined whether this modification of the intrinsic pathway was capable of acting in a synergistic manner with TRAIL to induce apoptosis in partially TRAIL resistant TCCB cells. Therefore, TRAIL (300 ng/ml) was added to T24, RT-112 and 253J cells that were untreated or treated with ASO-Bcl-2, and/or ASO-Clus or MM-control for 4 hours with MTT and/or live/dead assay performed 20 hours later. ASO-Bcl-2 or ASO-Clus alone significantly potentiated TRAIL induced death in T24 cells (figs. 8 and 9). Combined ASO treatment also augmented TRAIL induced death relative to TRAIL only but with an effect halfway between the effects of individual ASO-Bcl-2 and ASO-Clus despite our observation that ASO-Bcl-2 increased Clus expression. This observation was possibly due in part to competitive transfection uptake, as was evident by the degree of the decrease in Bcl-2 protein caused by combined ASO-Bcl-2 plus ASO-Clus treatment. Similarly in RT-112 and 253-J cells ASO-Bcl-2 treatment showed the most dramatic effect in enhancing TRAIL induced CD ($p < 0.001$). However, ASO-Clus had a relatively lower effect, again suggesting no added benefit over that of ASO-Bcl-2 alone. Consistent with others[8] we observed that MM transfection caused slight increases in TRAIL induced CD. This phenomenon is thought to be due to the direct toxicity of phosphorothioated oligodeoxynucleotides on the cells.

Increased Activation of Apoptotic Signaling with Combined TRAIL and ASO Treatment in TRAIL Partially Resistant TCCB Cells

The anti-apoptotic function of Bcl-2 and Clus is thought to be mediated via the inhibition of mitochondrial depolarization and the release of cytochrome C, thereby blocking caspase-9 and/or caspase-3 activation. In search of the molecular factors responsible for observed changes in the mitochondrial apoptotic pathway in TCCB we performed Western blotting to analyze lysates from T24, RT-112 and 253J cells transfected with 1,000 nM ASO-Bcl-2 and/or ASO-Clus, and then treated with 300 ng/ml TRAIL. TRAIL induced autocleavage of caspase-9 was robustly increased by the 2 ASOs, delineating the role of Bcl-2 and/or Clus in this intrinsic pathway (figs. 10 to 12). Furthermore, ASO

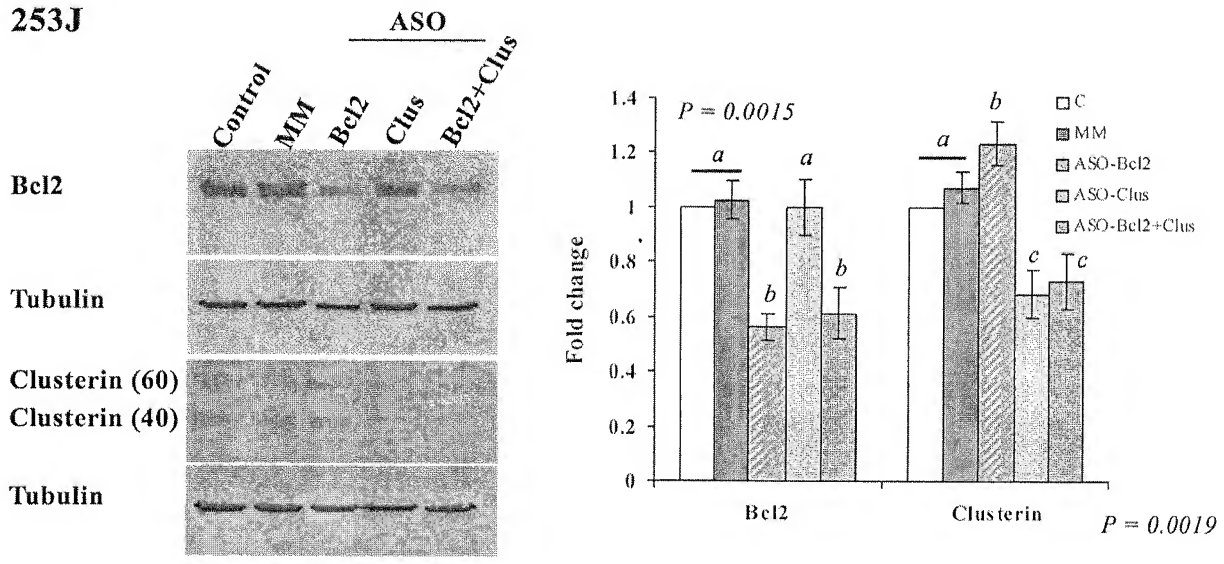

Figure 7. Protein expression of Bcl-2 and Clus after combined antisense therapy in TCCB cells. 253J cells were transfected for 4 hours with 1,000 nM ASO-Bcl2 or ASO-Clus singly or in combination as ASO-Bcl2 plus Clus, followed by additional 20-hour culture in fresh growth medium. Whole cell extracts were analyzed for Bcl-2 and Clus by Western blot. Graph shows quantitative analysis of expression of each protein after normalization to tubulin. Bars represent mean ± SE values of at least 3 separate experiments. *C*, control. *a* to *c*, significantly different.

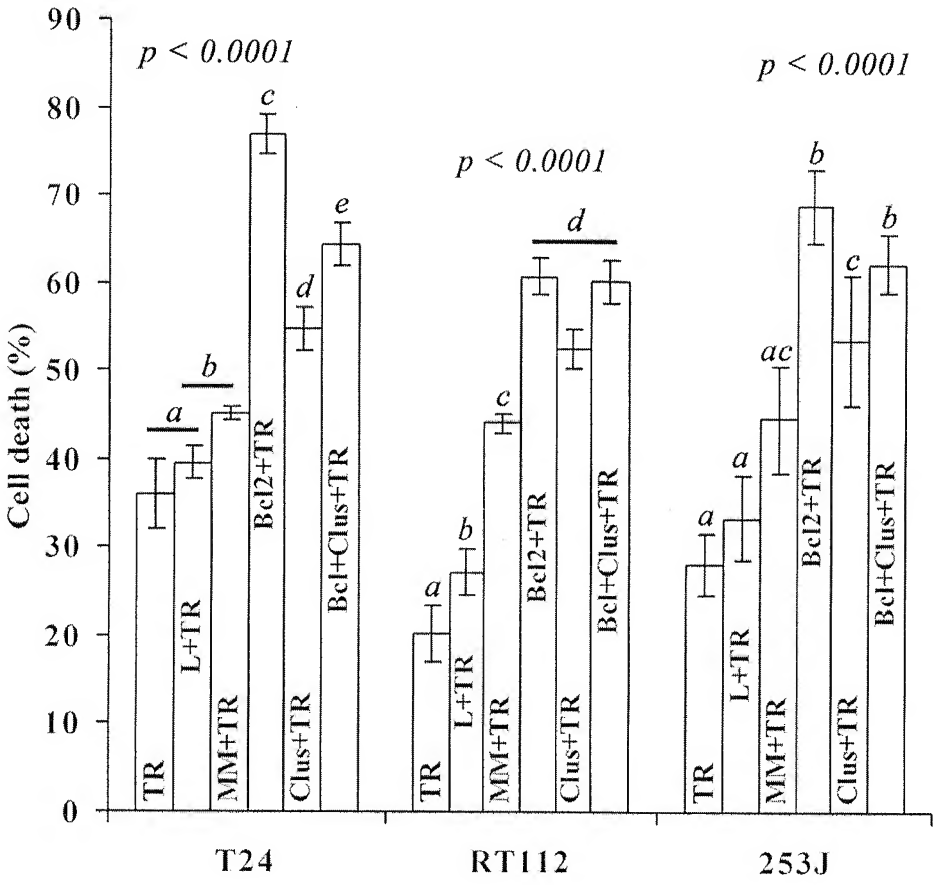

Figure 8. Response of TCCB cells to antisense treatments in combination with TRAIL (*TR*). Cells were transfected with 1,000 nM ASO-Bcl-2, and/or ASO-Clus or MM oligonucleotides for 4 hours. Medium containing transfection reagents was removed and replaced with fresh culture medium with additional 20-hour incubation. ASO transfected cells were treated with 300 ng/ml TRAIL. After 4 hours therapeutic medium was removed and replaced with fresh growth medium. Cells were incubated for additional 20 hours, during which MTT assays were performed. Results are shown as mean ± SE of at least 3 independent experiments. *L*, Lipofectin reagent. *Bcl2*, antisense to Bcl-2. *Clus*, antisense to Clus. *a* to *e*, significantly different.

potentiated TRAIL induced cleavage of downstream caspase-3 and DFF45, consistent with increased CD response in the same cell lines.

DISCUSSION

The DR ligand TRAIL has been implicated in the TCCB response to intravesical BCG immunotherapy.[2] In addition, it is considered a highly promising therapeutic agent for a wide range of other human malignancies. Despite its well described tumor selective pro-apoptotic properties monotherapeutic approaches with TRAIL are not that effective for activating apoptosis due to the acquired resistance of many TCCB cells to TRAIL. The development of TRAIL resistance by cancer cells is in part due to defects in the activation of the apoptotic signaling machinery downstream of surface receptor binding, including caspase-8 mediated Bid cleavage (which normally activates the mitochondrial pathway), caspase-8 mediated activation of caspase-3 or the TRAIL induced lysosomal pathway via c-Jun N-terminal kinase activation of Bim to permeabilize the lysosomes and engage the mitochondrial pathway.[9] We reasoned that increasing Bid activation or decreasing the inhibition of mitochondrial apoptotic signaling might reactivate TRAIL sensitivity in partially resistant TCCB cells. This was based on our observation that most TCCB cells in our panel underwent type 2 (mitochondrial dependent) DR apoptotic death (data not shown).

In many tumors Bcl-2 or other pro-survival relatives are often over expressed. Bcl-2 over expression in bladder cancer correlates with superficial TCCB recurrence and progression[10] and a poor prognosis of invasive cancer,[6,11] and it is a factor in patient responses to chemotherapy or radiotherapy.[12] Although some previous studies have failed to demon-

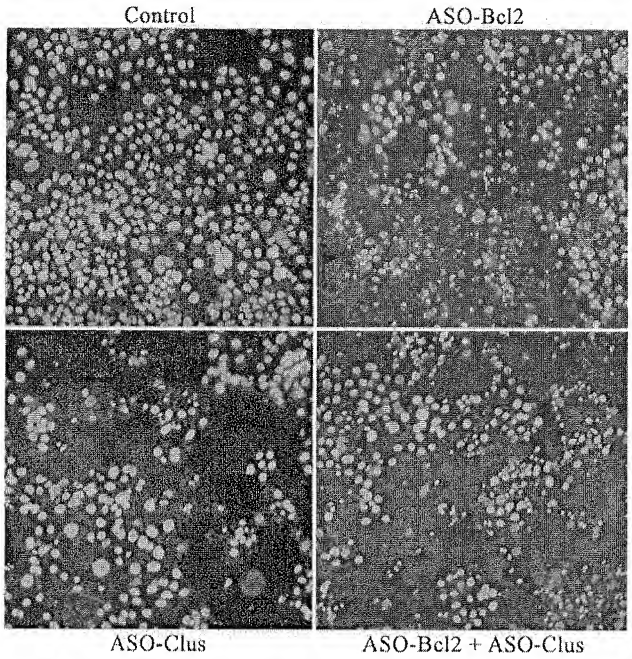

Figure 9. Response of TCCB cells to antisense treatments in combination with TRAIL. Cells were transfected with 1,000 nM ASO-Bcl-2, and/or ASO-Clus or MM oligonucleotides for 4 hours. Medium containing transfection reagents was removed and replaced with fresh culture medium with additional 20-hour incubation. ASO transfected cells were treated with 300 ng/ml TRAIL. After 4 hours therapeutic medium was removed and replaced with fresh growth medium. Cells were incubated for additional 20 hours, during which RT-112 cells were stained with 7.5 μM live cell nucleic acid probe Syto 16 (green) and 2.5 μM dead cell stain propidium iodide (red) for live/dead assay.

strate a role for Bcl-2 in protecting cells from TRAIL mediated apoptosis,[13,14] others have shown that it inhibits TRAIL induced or cisplatin induced CD.[15] In addition, Bcl-2 down-regulation may potentiate TNF and other chemotherapeutic agent mediated CD.[16] Furthermore, inhibitors of Bcl-2 or Bcl-xL are among the novel molecules that have recently been tested as reactivators of the mitochondrial apoptotic pathway in many cancer cells.[17,18] Therefore, the approach to inhibit the expression of these proteins is becoming obligatory as a novel therapy for future TCCB treatment.

In our study we used clinically available ASO methodology to down-regulate Bcl-2 expression to potentially activate the mitochondrial apoptotic pathway via the activation of Bax/Bak and the release of cytochrome C. Interestingly combination treatments of ASO-Bcl-2 and TRAIL significantly enhanced CD in partially TRAIL resistant TCCB cells. This augmentation of the TRAIL effect was associated with robust increases in activated caspase-9 and 3, and DFF45, consistent with apoptotic signaling. Upon exposure to TRAIL procaspase-9 and 3, and DFF45 were cleaved and rapidly depleted from the partially resistant TCCB cells (figs. 10 to 12). However, upon ASO-Bcl-2 treatment there was sustained activation of these proteins, presumably through a mechanism involving caspase-8 dependent cleavage of Bid, thus demonstrating the critical role of mitochondria in TRAIL mediated apoptosis.

Clinically Clus is frequently over expressed in various human malignancies, in which it correlates with tumor progression and resistance to cancer therapies, and has been shown to mediate tumor cell resistance to TRAIL and other chemotherapeutic agents by interfering with Bax activation in the mitochondria.[4,19] Clus silencing using ASOs such as OGX-011 has been attempted in patients with prostate cancer based on the enhanced apoptotic effect with conventional therapeutic modalities observed in prostatic cancer models.[20] Consistent with the study by Sallman et al in prostate cells,[4] we noted that ASO-Clus to a certain extent enhanced TRAIL mediated apoptosis in TCCB cells that were partially resistant to TRAIL. In addi-

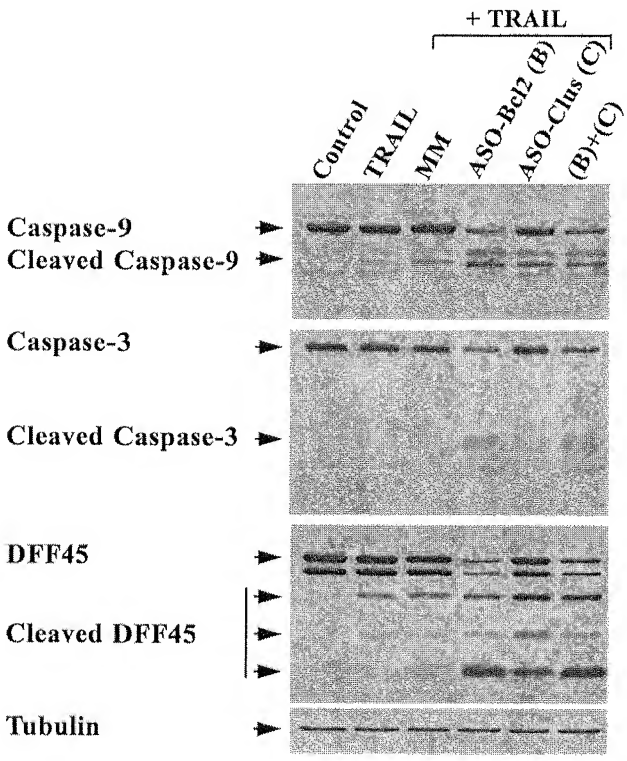

Figure 10. Representative blots show response of T24 TCCB cells to antisense treatments in combination with TRAIL. Cells were transfected with 1,000 nM ASO-Bcl-2, and/or ASO-Clus or MM oligonucleotides for 4 hours. Medium containing transfection reagents was removed and replaced with fresh culture medium with additional 20-hour incubation. ASO transfected cells were treated with 300 ng/ml TRAIL. After 4 hours therapeutic medium was removed and replaced with fresh growth medium. Cells were incubated for additional 20 hours. Whole cell extracts were analyzed for apoptosis signaling proteins by Western blot.

tion, we observed that ASO-Bcl-2 increased Clus expression in some cells, providing a further rationale for combination therapy. However, when ASO-Bcl-2 and ASO-Clus were combined, although there was a decrease in the levels of the 2 proteins, the expected synergy in enhancing sensitivity to TRAIL was not observed. In fact, in most cell lines the effects were found to be even lower than those of ASO-Bcl-2 transfection alone. This might be explained in part by the potential competitive transfection uptake of ASO-Bcl-2 and ASO-Clus. Overall our data suggest that down-regulation of Bcl-2 by ASO-Bcl-2 has the most potent impact on increasing TRAIL sensitivity and Clus expression may also have a role in TCCB TRAIL resistance.

CONCLUSIONS

We report that Bcl-2 or Clus inhibition alone can augment TRAIL induced apoptosis and reactivate TRAIL DR extrinsic signaling via increased activation of the intrinsic caspase pathway with an ultimate increase in apical caspase-3 and DFF45 in TCCB cells. Future combination studies are needed of the inhibition of other anti-apoptotic pathways and more effective strategies to down-regulate Bcl-2 and Clus expression.

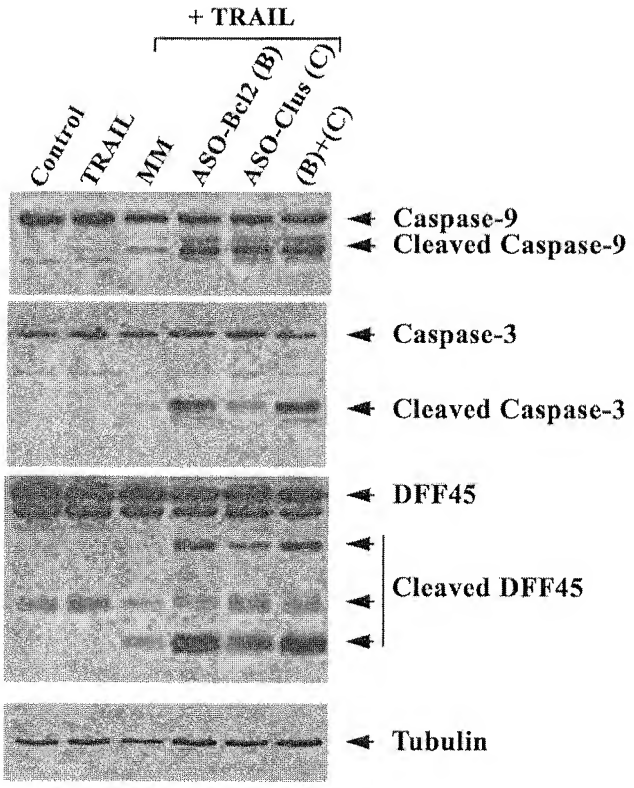

Figure 11. Representative blots show response of RT112 TCCB cells to antisense treatments in combination with TRAIL. Cells were transfected with 1,000 nM ASO-Bcl-2, and/or ASO-Clus or MM oligonucleotides for 4 hours. Medium containing transfection reagents was removed and replaced with fresh culture medium with additional 20-hour incubation. ASO transfected cells were treated with 300 ng/ml TRAIL. After 4 hours therapeutic medium was removed and replaced with fresh growth medium. Cells were incubated for additional 20 hours. Whole cell extracts were analyzed for apoptosis signaling proteins by Western blot.

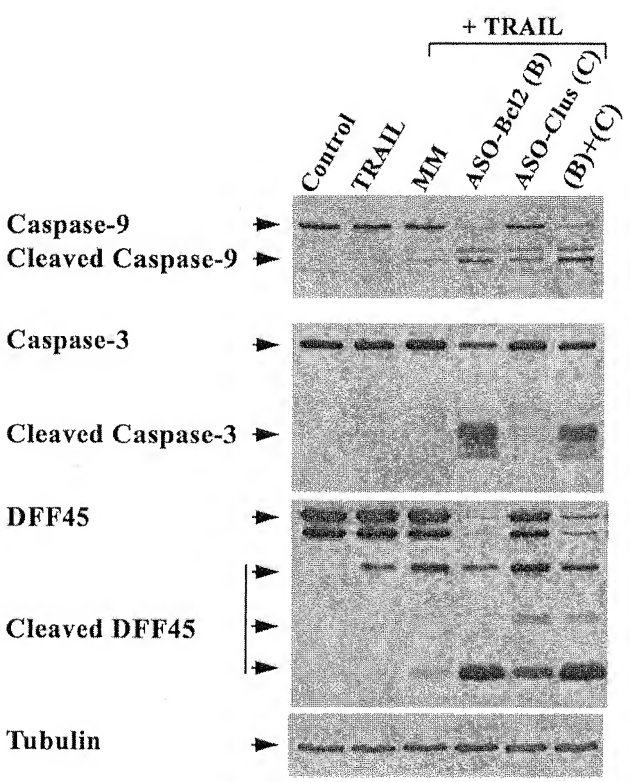

Figure 12. Representative blots show response of 253J TCCB cells to antisense treatments in combination with TRAIL. Cells were transfected with 1,000 nM ASO-Bcl-2, and/or ASO-Clus or MM oligonucleotides for 4 hours. Medium containing transfection reagents was removed and replaced with fresh culture medium with additional 20-hour incubation. ASO transfected cells were treated with 300 ng/ml TRAIL. After 4 hours therapeutic medium was removed and replaced with fresh growth medium. Cells were incubated for additional 20 hours. Whole cell extracts were analyzed for apoptosis signaling proteins by Western blot.

ACKNOWLEDGMENTS

Dr. H. B. Grossman, M. D. Anderson Cancer Center, Houston, Texas provided the UMUC-3, 6, 9 and 14 cell lines. Dr. M. Gleave, University of British Columbia, Vancouver, British Columbia, Canada provided phosphorothioated clusterin, and the RT-4, 253J and T24 cell lines. Dr. Y. Fradet, University Laval, Quebec City, Quebec, Canada provided the RT-112 and MGH-U3 cell lines. Oncogenex provided Bcl-2 ASO.

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